Annex B11.2

Real-time RT-PCR Assays for the Detection of Measles Virus (MeV) N Gene RNA and Human RNase P mRNA (a cellular reference gene) using the ABI 7500 Real-Time Thermocycler

This is an updated version of the measles real-time RT-PCR protocol (Annex B11). It has an 'updates' paragraph and the updates are highlighted in blue font.

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Purpose

The following protocol is to be used to detect RNA of measles virus (MeV) for diagnostic purposes. Primers and control RNA are supplied by CDC as a kit.
For these procedures, RNA is extracted from a clinical sample or from cell culture (See RNA Extraction protocol). If samples give a positive result in the real-time RT-PCR assays, the RNA may be tested in a measles genotyping RT-PCR assay to amplify the target for sequence analysis and genotyping (See Measles Genotyping RT-PCR protocol).

Important: This is a general protocol for use with the measles real-time RT-PCR kit supplied by CDC. Please check the package insert of the kit for updates to the protocol.

A master mix worksheet and plate layout are provided in a separate file.

Updates: **Changes to the previous version are in blue font.** Changes made to this protocol include:

1. Previous versions recommended testing samples in triplicate wells. In this protocol, samples are tested in duplicate wells. This will save reagent but may require more repeat tests if the duplicate wells produce discordant results.
2. Recommended expiration date for working solutions of primer/probe mixes
3. Recommended assay controls
4. Equipment set-up now includes separate targets for measles and RNase P
5. Verification that the threshold is set to the appropriate location and instructions for changing it as needed.
6. The assay controls section was expanded to include positive extraction controls and monitoring of the positive PCR controls.
7. The sample data quality control section was expanded to define retest conditions
8. Interpretation of results
9. Troubleshooting section now included

Reagents and materials needed-Listing of manufacturer does not imply endorsement by CDC or the US government

- 70% ethanol
- Aluminum foil
- Gloves
- Lab coat
- MicroAmp optical 96-well reaction plate with barcode (Life Technologies #4306737 or #4346906)
- Nuclease-free water (e. g. Life Technologies #AM9937)
- Optical adhesive cover (Life Technologies #4311971)
- RNase inhibitor 2000 units (e.g. Life Technologies N8080119)
- RNaseZap or similar (Sigma, # R2020-250ML)
- Sterile 1.5 ml microcentrifuge tubes
- SuperScript III Platinum OneStep qRT- PCR Kit (Life Technologies, #11732-020)

Equipment needed

- -70ºC and -20ºC freezers
- ABI 7500 real-time PCR System or another real-time PCR system
- Bucket with ice
- Centrifuge with holder for 96 well plates or plate spinner
- Class II biological safety cabinets (BSC) or PCR workstations with UV light designated for PCR set up
- Microcentrifuge
- PCR plate holder
- Micropipettors and sterile pipette tips with aerosol-resistant filters
- Vortex
- Water bath

Recommendations for working with RNA

- Use dedicated equipment, rooms and biosafety cabinets for all pre-PCR procedures. Post-amplification analysis and processing should be performed in a separate room using dedicated equipment. Do not share equipment (including lab coats) between pre-PCR and post-PCR procedures. Wear gloves throughout experiments to prevent contamination from RNases found on human hands.
- Change gloves after touching skin (e.g. your face), doorknobs, and common surfaces.
- Have a dedicated set of pipettors that are used solely for RNA work.
- Use filter tips and tubes that are tested and guaranteed to be RNase-free.
- Use RNase-free chemicals and reagents.
- Reduce RNase contamination by cleaning tube racks, pipettors, and the work surface of the PCR hood with 70% ethanol and with RNaseZap wipes.
- Reduce DNA contamination with UV light exposure for 15 minutes.

Kit contents

The MeV real-time RT-PCR kit consists of two boxes.

Box 1 should be opened in the BSC used for preparation of the master mix. It contains:

- MeV primer/probe mix: One tube with 100 µL of a mix of primers and probe for MeV real-time reactions. Content needs to be diluted before use (see below).
- RNase P primer/probe mix: One tube with 50 µL of a mix of primers and probe for RNase P real-time reactions. Content needs to be diluted before use (see below).

Box 2 should be opened in the BSC used for addition of samples to the master mix. It contains:

- MeV control RNA (high concentration control). Contains synthetic MeV RNA (MeV-N3in) and total human RNA. Content is dried and needs to be rehydrated and diluted before use (see below). This control can be used for both MeV and RNase P reactions.
- MeV control RNA (low concentration control). Contains synthetic MeV RNA (MeV-N3in) and total human RNA. Content is dried and needs to be rehydrated and diluted before use (see below). This tube contains less synthetic MeV RNA than the high control, but the same amount of total human RNA. This control can be used for both MeV and RNase P reactions.
- 2 mL TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.0) for rehydration of controls.

Information about primers and probes

- Primer and probe stock solutions are stored at –20°C. Keep probes protected from light.
- Probes are labeled at the 5' terminus with a fluorescent reporter dye, 6-carboxy-fluorescein (FAM), and at the 3' terminus with a non-fluorescent quencher, black hole quencher-1 (BHQ1).

- Final concentration in the reaction mix:
  - MeV primers and RNase P primers: 300 nM
  - MeV probe: 250 nM
  - RNase P probe: 100 nM
- MeV primer sequences
  - Forward Primer (MVN1139-F): 5' TGG CAT CTG AAC TCG GTA TCA C 3'
  - Reverse Primer (MVN1213-R): 5' TGT CCT CAG TAG TAT GCA TTG CAA 3'
  - Probe (MVNP1163-P): 5' FAM CCG AGG ATG CAA GGC TTG TTT CAG A BHQ 3'
- RNase P primer sequences
  - Forward Primer (HURNASE-P-F): 5' AGA TTT GGA CCT GCG AGC G 3'
  - Reverse Primer (HURNASE-P-R): 5' GAG CGG CTG TCT CCA CAA GT 3'
  - Probe (BHQ1 HURNASE-P): 5' FAM TTC TGA CCT GAA GGC TCT GCG CG BHQ1 3'

Preparation of working solutions of primer/probe mixes

Primer/probe mixes are supplied as 10x (10-fold) concentrated stocks. It is necessary to prepare a working solution of each mix before setting up a real-time RT-PCR reaction. Store diluted primers and probes at -20°C. Wrap tubes in aluminum foil to protect from light. Aliquots should be thawed no more than three times. Discard working solution after three months.

- MeV primer/probe mix: Mix 10 µL stock solution with 90 µL nuclease-free water. Vortex.
- RNase P primer/probe mix: Mix 10 µL stock solution with 90 µL nuclease-free water. Vortex.

Preparation of control RNA stocks

Control RNAs are supplied as dried RNA. It is necessary to rehydrate these controls before the first use of the kit. Always work with RNA on ice. Do not work with control RNAs in the same PCR hood where master mix preparation is carried out.

1. To each tube (high and low control RNA) add 100 µl nuclease-free TE (supplied in kit) and vortex for 15 seconds.
2. Heat tubes to 50°C for 15 minutes in a water bath then vortex for 15 seconds. Spin briefly to collect.
3. Prepare aliquots of 10 µl each and store at -70°C. Each aliquot should be thawed only once to make a working solution (see below).
4. Label each tube with lot number, date of rehydration, and indicate that it is 10X stock.

Preparation of working solutions of control RNAs

1. Thaw one tube with 10 µL high control RNA stock and one tube with 10 µL low control RNA stock.
2. To each tube, add 90 µL nuclease-free TE (supplied in kit). Vortex. Spin briefly to collect.
3. Prepare 10 aliquots of 10 µL each and store at -70°C.

4. Label each tube with lot number, date of preparation, and indicate that it is working stock.
5. Use one aliquot for each real-time RT-PCR assay. Discard leftover working solution.

Sample Preparation

RNA samples (extracted from a clinical sample or from cell culture, see RNA extraction protocol) are stored at -70°C. Usually, RNA is extracted from one 25 cm^2 flask of infected cells or from 100-200 µL of clinical material. Most of the extraction protocols yield 40-50 µL of RNA. For the assay, 2.5 µL of sample are used per reaction. The volume of RNA can be increased, but this will not significantly improve the sensitivity. Addition of different volumes requires adjustment of added nuclease-free water to result in a final volume of 25 µL.

Assay Controls

- Every RNA sample should be tested in parallel with the MeV primer/probe and the RNase P primer/probe. The purpose of the RNase P control reaction is to monitor the integrity of the RNA.
- The following standards and controls should be run on each plate as indicated on the Measles Real-time Plate Layout. They must be included in master mix calculations for each primer/probe set:
    - Negative controls
        - NTC: add 2.5 µL per well nuclease-free water instead of RNA to the wells labeled MeV NTC and RNP NTC
        - Negative extraction control obtained by extraction of water or cell culture medium. It is recommended to test the negative extraction control with the MeV and the RNase P primer set.
    - Positive controls
        - Positive extraction control: extraction of uninfected cells (positive signal in RNAse P assay). This control should be tested with the RNase P primer set. Aliquots from a pool of previously tested samples that were negative for measles but positive for RNase P can also be used.
        - Two control RNAs (high control and low control)
            - Rehydration and dilutions should be done separately from set up of master mix.
            - Either one of the controls can also be used as RNase P controls.
            - Add 2.5 µl/well of each control RNA.

Preparations for assay set up

- Thaw kit reagents: 2X reaction mix, ROX, and primer/probe mix and briefly vortex.
- Always keep enzymes on ice.
- Spin down all reagents (including enzymes) in microcentrifuge and keep on ice until ready to dispense.
- Thaw RNA samples and keep on ice during assay set-up.
- Record the date when reagents were opened on Measles Real-time Master Mix worksheet.

Assay Protocol

1. Determine the number of reactions (n) based on the number of RNA samples to be tested and the format of the Measles Real-time Plate Layout.
    a. All reactions with the measles primer set may be tested in duplicate wells: clinical samples, NTC, high and low control, negative extraction control.
    b. All reactions with the RNase P primer set may be tested in single wells: clinical samples, NTC, RNase P positive control (either high or low control), negative and positive extraction controls.

Prepare excess reaction volume (n + 1) for each primer/probe set to allow for pipetting errors.
Calculating the number of reactions:

a. For the MeV primer set, the number of reactions is 2 times the number of samples plus 6 for the real-time run controls (NTC, high control, low control), plus 2 for the negative extraction control plus 1 to allow for pipetting losses.
b. For the RNase P primer set, the number of reactions is the number of samples plus 1 for the positive extraction control, plus 1 for the negative extraction control plus 2 for the real-time run controls (NTC and either high or low control) plus 1 to allow for pipetting losses.

Example: If there are 4 samples, make a master mix for 17 reactions with the MeV primer set:

- 4 samples measured in duplicate=8 reactions
- 2 NTCs
- 2 reactions high control RNA
- 2 reactions low control RNA
- 2 reactions for the negative extraction control
- 1 extra to allow for pipetting losses

Make a master mix for 9 reactions with the RNase P primer set:

- 4 samples
- 1 positive extraction control
- 1 negative extraction control
- 1 NTC
- 1 reaction with either high or low control RNA
- 1 extra to allow for pipetting losses

2. Enter the ID# of the sample(s) on the Measles Real-time Plate Layout.
3. Enter the total number of reactions in the master mix section on the Measles Real-time Plate Layout to determine volumes of each reagent to be added. There are separate calculations for the measles primers and the RNase P primers. See the example calculation for 1 sample on page 10.
4. In the BSC dedicated for master mix preparation, for the MeV primer/probe set, add the first 4 reagents (nuclease-free water through ROX reference) to a pre-chilled 1.5 mL microcentrifuge tube. Invert, briefly centrifuge, and keep on ice.
5. Add RNase inhibitor and enzyme mix to master mix tube. Vortex, centrifuge briefly and chill on ice.
6. In another 1.5 mL microcentrifuge tube, repeat steps 4 and 5 for the RNase P master mix.
7. Dispense 1 reaction volume of master mix into appropriate wells according to the Measles Real-time Plate Layout, using a new tip for each master mix. Reaction volume is 22.5 µL/well for MeV and RNase P.
8. Add 2.5 µL/well nuclease-free water (NTC) to the designated wells on the Plate Layout.

9. Proceed to a separate BSC designated for template addition. Add extraction controls, sample RNA, high or low positive controls as indicated on the Measles Real-time Plate Layout, using a new tip for each well. Sample volume is always 2.5 µL/well. The total volume in each well should now be 25 µL.
10. Seal plate with optical adhesive cover.
11. Centrifuge the sealed plate at 1500 rpm for 1 minute at room temperature.

Assay Run

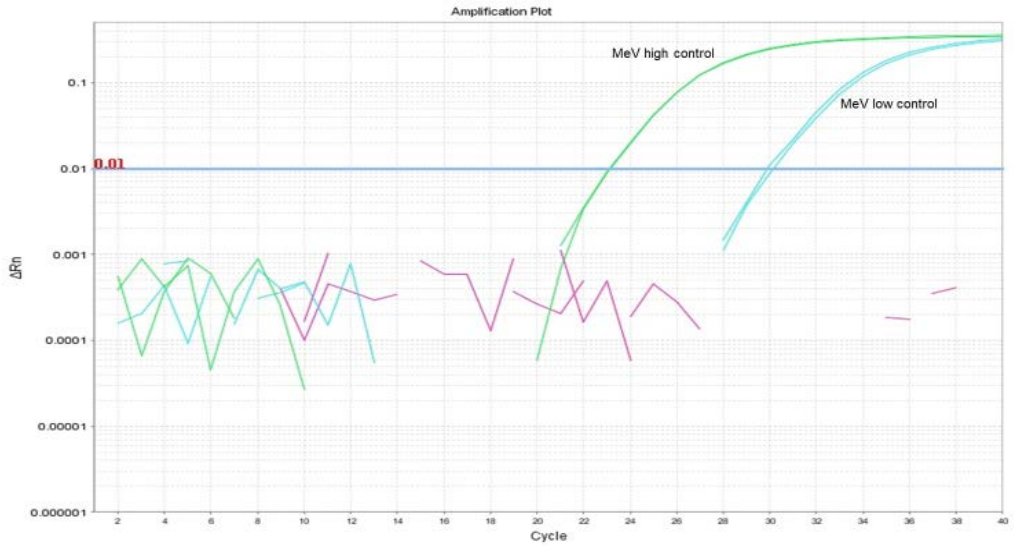
1. Launch software by double-clicking the 7500 software icon on the desktop. The following instructions are for v2.0.4 of the software.
2. Turn on the 7500 thermocycler – connection is usually automatic.
3. Select Advanced Set-Up.
4. The Experiment Properties screen will open. Name the experiment (e.g. date and initials). The settings should be 7500 (96 wells), Quantitation-Standard Curve, TaqMan reagents, Standard Ramp Speed.
5. Go to Plate Setup: Define Targets and Samples. Enter the name of each sample to be tested by clicking on Add New Sample. Include extraction controls.
6. Go to Define Targets. Under Target Name, change the name of 'Target 1' to 'MeV'. The target for measles is FAM and the quencher NFQ-MGB (Blackhole Quencher). The target is the fluorescent dye that the instrument will detect. Click on Add Target. Name the new target 'RNase P'. The target for RNase P is FAM and the quencher NFQ-MGB (Blackhole Quencher). Choose different colors for the MeV and RNase P targets.
7. Go to Assign Targets and Samples. Using the Plate Layout as a guide, highlight all the wells that will contain MeV reaction mix. Under Assign Target(s) to the Selected Wells check the box for the MeV target. Then highlight all the wells that will contain RNase P reaction mix. Under Assign Target(s) to the Selected Wells check the box for the RNAse P target. The wells will be labeled with the colors representing the two different targets. All the marked wells will display 'U' for 'unknown'. Do not highlight empty wells.
8. To define standards, highlight the wells with the MeV target that are for the high control. Under 'Assign Targets', click on 'S' for standard. Under 'quantity', fill in 100000 (10^5). Repeat for the low control with 1000 (10^3) as quantity. To define the RNase P control, highlight the well, click on 'S' for standard. It is not necessary to change the quantity.
9. To define the NTC, highlight the wells with the measles and RNase P water control. Under 'Assign Targets', click on 'N' for NTC.
10. To assign samples, highlight the 2 wells that contain the MeV master mix for the first test sample (e.g. sample 1). Under 'Assign samples to the Selected Wells' check the box for sample 1. Repeat for the well that contains sample 1 and RNase P master mix. Repeat this process for each sample. Each well in use should now contain a target, a task (NTC, standard or unknown) and a sample name.
11. Make sure ROX is listed as the passive dye.
12. Go to Run Method. Set the Sample Volume to 25 µL. Set the step parameters (time/temp) on the thermal profile to the following:
    - RT Step: 48ºC /30 minutes
    - Activation: 95ºC /5 minutes
    - PCR (40 cycles): 95ºC /15 seconds, 60ºC /1 minute
13. Select File > Save As and save file in user folder using a standard format: (e.g. Date_initials.eds)
14. Open the door of the 7500 by pushing in the indentation on the front.
15. Place a plate into the instrument tray. Orient the A1 well on the plate with the A1 position on the instrument tray.

16. Select Start Run. Under Run, select Amplification Plot to monitor the run, which should be complete in approximately 2 hours. The screen will show how much time is left until the end of the run.
17. At the end of the assay, the green Analyze button will appear.
18. Click on Save to save your data.

Checking the threshold

1. After the run has finished, click on Analyze. The program will calculate an automatic threshold.
2. The results will appear. Under Plot Settings, verify that the graph type is 'log'.
3. Below the amplification curve, under Options, choose the MeV target. You will only be able to see the amplification curves from the wells containing MeV primer mix.
4. Highlight only the wells with the MeV high and low control and the MeV NTC. Do not include the sample wells.
5. Check that the threshold is in the lower third of the exponential phase of the positive controls, where the duplicates for the control are closest to each other. Figure 1 below shows an example. In rare cases, the program may not choose the best place for the threshold. It may be necessary to manually move the threshold:
    a. Under Options, uncheck Threshold Auto. Move the cursor to the threshold line. The cursor will change to a hand symbol. Click and drag the threshold to the appropriate place in the lower third of the exponential phase of the positive controls and above the signal for the NTC. Every time the threshold is moved, the Ct values for the samples will be automatically recalculated.
    b. Save the file under a new name because the program will not retain the information for the new threshold.
6. Repeat steps 3-5 for the RNP threshold.

Figure 1: Amplification plot with high and low control curves.



Assay Quality Control

Visual inspection of the amplification plots is important. Positive samples and controls should show exponential amplification curves. Negative samples and controls should show no evidence of amplification.

Checklist for quality control: A real-time RT-PCR assay is valid if:

- The threshold is located in the exponential phase of the standard amplification curves.
- The NTCs for MeV and RNase P are Undetermined (negative).
- The negative extraction control is Undetermined (negative).
- The positive extraction control is positive.
- The Ct values for the duplicate samples of the MeV high and low controls fall within the range defined in the measles real-time kit package insert.
- The Ct value for the single sample of the RNase P control falls within the range defined in the measles real-time kit package insert.

If any of these criteria are not fulfilled, the assay is invalid.

If the negative extraction control is positive, this indicates contamination during the RNA extraction. The RNA extraction should be repeated.

If the positive extraction control is negative, this indicates problems with the RNA extraction. The RNA extraction should be repeated.

If PCR controls (NTC, high or low control, RNAse P positive control) are negative or not in range, the assay must be repeated.

Monitoring real-time RT-PCR assay over time: Ct values for at least one of the MeV positive controls should be recorded for subsequent runs. It is also useful to track the Ct value of the positive extraction control. After several assays, a typical range of Ct values for the control should be determined and used for the analysis of subsequent runs. Changes in these values may indicate problems. It is recommended to use Levy-Jennings charts to track the performance of the control.

Important: the range of Ct values for the high and low positive controls may vary between different lots of the Measles Real-time RT-PCR kit. Please check the package insert for updates.

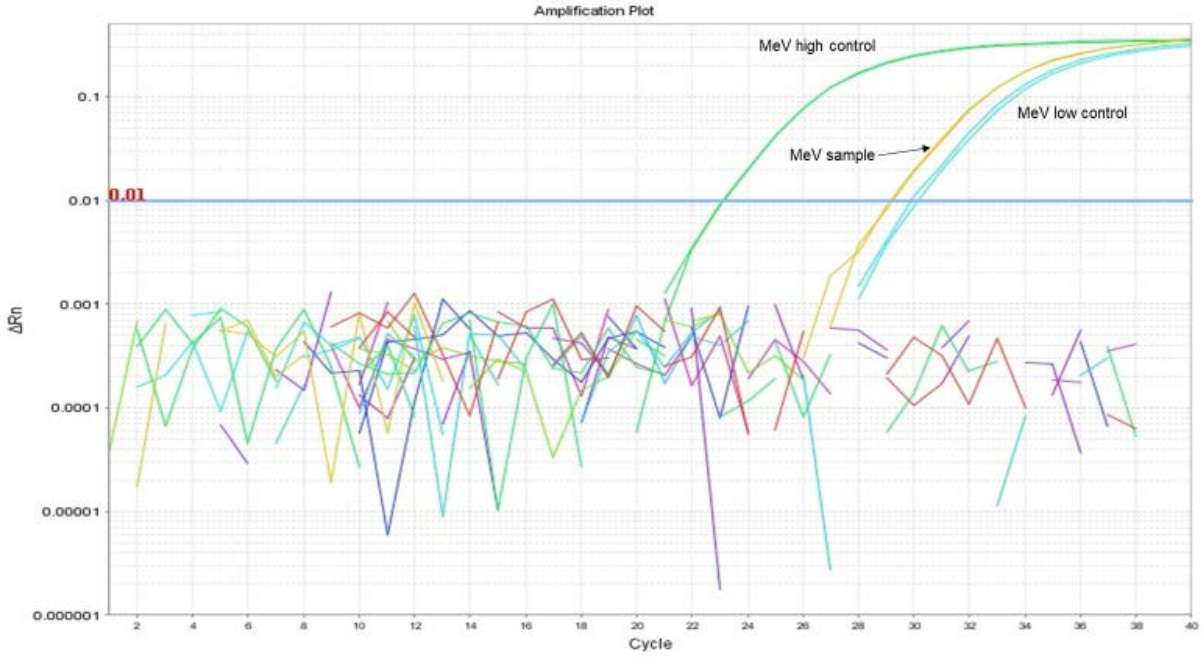
Sample Data Quality Control

After the assay has been determined to be valid, quality control must be performed for each sample.

1. Check the Multicomponent Data Pane icon on all samples with Ct values < 40 to confirm true amplification as indicated by a rise in FAM fluorescence.
2. Some wells may be highlighted by yellow flags. Click on the QC Summary Pane to see the reason why the wells were flagged. For example, if a sample well did not produce a signal, the program will flag this with No amplification. However, if the sample is negative (for example the negative extraction control), a lack of amplification is the expected result and not a quality control issue.
3. Samples must be retested if:
    a. replicates are discordant (>2 Ct apart)
    b. one replicate is < 38 and the other is ‘undetermined’
    c. one is < 38 and the other is > 38 and < 40
    d. the interpretation of the test is ‘inconclusive’ (see below)

Figure 2 below shows the 2 positive controls and a typical positive test sample.

Figure 2: Amplification plot with standards and typical sample



Interpretation of results for MeV, RNase P

MeV	RNaseP	Interpretation
Ct <38	Ct <40	Positive
Ct <38	Undetermined	Positive
38≤Ct<40	Ct < 40	Inconclusive
38≤Ct<40	Undetermined	Inconclusive
Undetermined	Ct<40	Negative
Undetermined	Undetermined	Invalid

- If the PCR with the MeV primers/probe produced a Ct below 38, the sample is positive for measles. It does not matter whether the RNase P reaction is positive or negative.
- If the PCR with the MeV primers/probe produced a Ct between 38 and 40, the result is inconclusive for measles. It does not matter whether the RNase P reaction is positive or negative. The PCR must be repeated. If the repeated test gives the same result, the sample should be reported as inconclusive.
- If the result of the MeV PCR is Undetermined but the RNase P PCR produced a Ct below 40, the sample is negative for measles. The positive RNase P result indicates that enough RNA was extracted from the sample to allow amplification to occur.
- If the result of the MeV PCR and the RNase P PCR are Undetermined, the sample is invalid. It is not possible determine a result, because the RNA quantity or quality were insufficient to allow amplification to occur. The test must be repeated. It is recommended to repeat the RNA extraction. If the repeated test gives the same result, the sample should be reported as invalid.

Exporting Data

To save the data to an Excel file: Click on Export, then

1. Under: select data to export, choose Results
2. Under: Select one file or separate files, choose One file
3. Choose a file name and browse to the location where the file should be saved.
4. Click Open, then click Start Export.
5. Close the Export Tool.

Master mix worksheet for measles primer/probe set

Component	Vol/rxn (µl)	# of rxns	Total vol (µl)
Nuclease-free water	7.2	1	7.2
2x reaction mix	12.5	1	12.5
MeV primer/probe mix	2	1	2
ROX reference	0.05	1	0.05
RNase inhibitor	0.25	1	0.25
SS III/Taq mix	0.5	1	0.5
RNA	2.5	xxxx	xxxx
Total	25		

Master mix worksheet for RNaseP primer/probe set

Component	Vol/rxn (µl)	# of rxns	Total vol (µl)
Nuclease-free water	7.2	1	7.2
2x reaction buffer	12.5	1	12.5
RNP primer/probe mix	2	1	2
ROX reference	0.05	1	0.05
RNase inhibitor	0.25	1	0.25
SS III/Taq mix (5U/µl)	0.5	1	0.5
RNA	2.5	xxxx	xxxx
Total	25		

Dispense master mix: 22.5 µl/well for both master mixes. Add 2.5 µl template

Troubleshooting

Problem	Possible causes
All reactions negative, including positive control	Component missing in master mix set up Wrong thermocycler settings Expired or inappropriately stored reagent
No amplification (i.e. negative) of a positive PCR control	High or low control RNA degraded or not added to wells
Amplification in a NTC well	Contamination of the reagents Contamination of the equipment (PCR block, pipettes) Contamination due to technician error during the PCR set up
Amplification in the negative extraction control	Contamination due to technician error during the extraction procedure
No amplification (i.e. negative) of the positive extraction control	Error during the RNA extraction Extraction control sample degraded or pipetting error